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# Free plasma testosterone levels during the normal menstrual cycle

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ABSTRACT. This report describes the free or unbound testosterone levels in ten normal females during the menstrual cycle, using a simplified technique of equilibrium dialysis of undiluted plasma. Total testosterone concentration fell progressively during the menstrual cycle, whereas the percent free testosterone increased from the follicular to the luteal phase. Free testosterone levels also fell but only significantly at the late luteal phase. For comparison two patients with anovulatory cycles were evaluated. Progesterone displacement of endogenous testosterone from its binding protein(s) was suggested by *in vitro* studies.

## INTRODUCTION

It has been known for some time that the physiological effect of a given circulating sex steroid is not correlated with the total plasma concentration, but rather with that fraction of the total which is not bound to the high affinity sex hormone-binding globulin (SHBG) and to the low affinity serum albumin, with the suggestion that the latter may supplement the free pool of hormone within tissues because of the rapid dissociation of steroids from albumin (1).

Using a simple and precise technique of equilibrium dialysis for the direct measurement of percentage free testosterone in plasma, we performed a paired study in ten normal females, collecting samples at several times during their menstural cycle. For comparison two patients without hormonal evidence of corpus luteum formation were also studied. Experimental data were also obtained from incubation of plasma samples with progressive amounts of progesterone.

## PATIENTS AND METHODS

Ten women with ages ranging from 24-40 yr with body weights within the normal range according to the Metropolitan Life Insurance tables, with regular menstrual cycles of 25 to 36 days, were studied. Cycles were considered to be ovulatory on the basis of basal temperature and plasma progesterone levels greater than 5 ng/ml in the luteal phase (2). Because of variation in the length of the individual cycles, the 4 blood samples collected in every subject were pooled using the "midcycle" day for departure (day 0 of cycle). Then the samples were considered to be collected from day - 9 to - 7 (considered the "early follicular phase" taking into consideration the estradiol and progesterone levels obtained in the samples — EF), from day — 3 to — 1 ("late follicular phase" — LF), from day +3 to +7 ("early luteal phase" — EL) and from day +8 to +14 ("late luteal phase" — LL).

Two women (subjects n. 11 and 12), 23 and 25 -year-old, nonobese, with probably anovulatory cycles presenting increasing estradiol concentrations at the expected luteal phase but progesterone levels uniformly low termined by an episode of vaginal bleeding after 32 and 31 days, respectively, were studied.

All subjects were informed about the nature of the procedure and written consent obtained in every one.

Total estradiol ($E_2$), testosterone (T), progesterone (P) RIAs were performed as described previously (3-5).

The validation of the total testosterone radioimmunoassay was satisfied by specific studies, evaluating the crossreactivity of the antiserum and by dilution test, comparing the reactivity of the plasma hormone with that of the standard. The antiserum used was prepared against testosterone 19-carboxymethylether-BSA, *having a high degree of specificity to testosterone* presenting only significant crossreaction with 5 $\alpha$-dihydrotestosterone (7%). We have obtained identical values for both male and female testosterone levels with and without chromatography. In the dilution test, the correlation coefficient between the dilution and the steroid concentration was highly significant ($r = 0.997$). The sensitivity of the standard curve for the testosterone RIA was 5 pg. The intra-and interassay coefficients of variation were 5.9% and 10.0%, respectively. Finally, the recovery was in the range of 88 to 109% of the steroid added to the steroid-free plasma (low and high pool).

Unbound or free testosterone was determined by equi-

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librium dialysis by the technique described by Kley, Bartmann and Krüskemper (6) in which dialysis tubing containing 1 ml of undiluted plasma was dialyzed against 10 ml phosphate buffer 0.05 M, pH 7.4 containing about 8,000 cpm 1,2 $^3$H testosterone (sp. activ. 40 Ci/mmol, obtained from New England Nuclear, Boston, MA, USA and purified on a Sephadex LH-20 column) in a 20 ml glass vial. The vials were kept in a shaking water bath at 37 C for 16 h. Under these conditions, equilibrium was reached after 10 h of incubation and remained unchanged up to 24 h. Samples of 1 ml from outside of tubing were taken for measurement of radioactivity and compared to 1 ml of the original buffer containing the radioactive testosterone (before dialysis).

The percent of free testosterone was calculated according to the formula (7):

$$\frac{\text{cpm/ml inside dialysis tube}}{\text{cpm/ml buffer predialysis} - \text{cpm/ml outside dialysis tube}} \quad 10$$

$$\% \text{ Free} = \frac{\text{cpm/ml outside dialysis tube}}{\text{cpm/ml inside dialysis tube}} \quad 100$$

The product of total testosterone concentration, as determined by RIA times the percentage unbound gives the concentration of unbound or free steroid.

Our method of calculation was compared to that presented by Kley et al. (6) in which the alteration occurring in the volume inside tubing is corrected by the difference in weight before and after dialysis. The comparison was done in 62 paired samples showing as value of 0.806 ($t_{0.05} = 2.000$), the correlation coefficient between the 2 methods being 0.9852, with a linear regression represented by the equation $x_{mod} = 0.9956\ y_{orig} + 0.1237$, indicating that both methods gave similar results. The coefficients of variation were: 6.2% intraassay and 7.2% for interassay (7).

To test the hypothesis that physiological levels of progesterone can displace endogenous testosterone from its binding proteins, plasma from patients nº 3 (LF), 4(EF), 5(EF) and 10 (LF) as well as from 2 pools obtained from normal and hirsute females respectively, were incubated for 24 hours at 37 C with a progesterone standard in buffer (1ng/ul) in amounts variable from 2 to 15 ng of steroid/ml of plasma. The relation between final progesterone concentration in plasma and correspondent % FT is indicated for each sample in Figure 1. Besides, for each plasma from the patients studied, the LL phase was evaluated again in the same assay incubated for the obtention of the regression line (Fig. 1).

*Statistical Analysis*

The data were analyzed by analysis of variance (ANOVA) (7) with the F statistic. The least significant difference (LSD) method was used when F was significant (8).

## RESULTS

The estradiol and progesterone levels measured in our normal subjects were indicative of ovulatory cycles, the values are shown in Table 1.

Table 1 indicates that plasma total testosterone (T) was significantly variable within the phases of the menstrual cycle (F = 4.01612), the comparison between pair of means indicating that the testosterone values were significantly lower in both luteal periods when compared to the follicular ones. However, no differences were noticed in the sampling within the follicular or luteal phases. Similarly, percent free testosterone (%FT) was variable within the menstrual cycle (F = 4.1679), the comparison between pair of means also showing higher values in the luteal periods in relation to follicular ones. Furthermore, no differences within the follicular or luteal phase were demonstrated.

Unbound or free testosterone concentration (FT) was also significantly variable within the menstrual cycle (F = 3.5367). The comparison between pair of means demonstrated that only in the late luteal phase were the levels significantly lower when compared to the early follicular phase. No differences were present within the follicular or luteal phases. Therefore, the FT remained relatively constant throughout the menstrual cycle except at the late luteal period with the lowest total T and highest percent of free hormone not compensating for the significant decrease in the calculated level of FT.

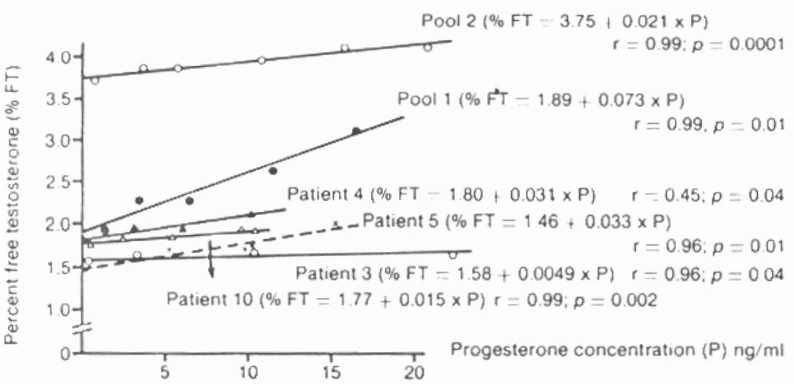


Fig. 1 - *Relation between Progesterone concentration and Percent Free Testosterone in Plasma samples.*

Table 1 - *Plasma estradiol, progesterone, total testosterone, percent free testosterone and free testosterone concentration in 10 normal women during the menstrual cycle and subjects with "anovulatory" cycles*

| Subjects no. | Plasma Estradiol (pg/ml) | | | | Plasma Progesterone (ng/ml) | | | | Plasma total testosterone (ng/ml) | | | | Percent Free Testosterone (%) | | | | Plasma Free Testosterone (pg/ml) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EF[1] | LF[2] | EL[3] | LL[4] | EF | LF | EL | LL | EF | LF | EL | LL | EF | LF | EL | LL | EF | LF | EL | LL |
| 1 | 43.9 | 69.9 | 155.7 | 149.3 | 0.16 | 0.13 | 1.92 | 12.71 | 0.571 | 0.591 | 0.484 | 0.342 | 1.31 | 1.38 | 1.58 | 1.95 | 7.49 | 8.16 | 7.65 | 6.68 |
| 2 | 67.0 | 77.6 | 111.3 | 143.8 | 0.43 | 0.50 | 1.80 | 9.09 | 0.307 | 0.274 | 0.270 | 0.202 | 1.80 | 1.90 | 2.00 | 2.05 | 5.53 | 5.21 | 5.40 | 4.14 |
| 3 | 59.8 | 102.6 | 129.5 | 177.5 | 0.30 | 0.36 | 6.00 | 22.21 | 0.330 | 0.248 | 0.266 | 0.275 | 1.40 | 1.57 | 1.55 | 1.68 | 4.62 | 3.89 | 4.12 | 4.62 |
| 4 | 37.1 | 99.8 | 92.7 | 120.5 | 0.16 | 0.17 | 4.62 | 6.08 | 0.471 | 0.446 | 0.314 | 0.286 | 1.78 | 1.76 | 1.66 | 1.94 | 8.36 | 7.85 | 5.21 | 5.55 |
| 5 | 59.5 | 150.2 | 138.1 | 167.3 | 0.24 | 0.84 | 3.69 | 9.76 | 0.510 | 0.464 | 0.415 | 0.291 | 1.45 | 1.60 | 1.94 | 1.73 | 7.40 | 7.42 | 8.05 | 5.03 |
| 6 | 65.4 | 175.8 | 126.3 | 195.4 | 0.20 | 0.21 | 2.84 | 6.20 | 0.500 | 0.453 | 0.260 | 0.233 | 1.30 | 1.20 | 1.50 | 1.48 | 6.50 | 5.71 | 3.90 | 3.45 |
| 7 | 77.7 | 95.0 | 183.0 | 210.0 | 0.46 | 0.50 | 2.74 | 5.99 | 0.502 | 0.450 | 0.330 | 0.307 | 1.56 | 1.54 | 2.01 | 2.12 | 7.83 | 6.93 | 6.63 | 6.51 |
| 8 | 65.0 | 90.0 | 119.2 | 139.4 | 0.32 | 0.35 | 1.78 | 10.69 | 0.440 | 0.400 | 0.302 | 0.301 | 1.72 | 1.70 | 1.97 | 2.02 | 7.58 | 6.80 | 5.93 | 6.12 |
| 9 | 45.0 | 81.3 | 91.2 | 108.0 | 0.30 | 0.40 | 3.16 | 15.00 | 0.689 | 0.600 | 0.533 | 0.342 | 1.20 | 1.18 | 1.40 | 1.50 | 8.27 | 7.08 | 7.46 | 5.14 |
| 10 | 30.0 | 82.0 | 166.9 | 257.6 | 0.21 | 0.47 | 1.11 | 9.57 | 0.352 | 0.395 | 0.274 | 0.286 | 1.76 | 1.77 | 1.90 | 1.93 | 6.20 | 6.99 | 5.21 | 5.52 |
| x | 55.0 | 102.4 | 131.4 | 166.9 | 0.28 | 0.39 | 2.97 | 10.73 | 0.467 | 0.432 | 0.315 | 0.287 | 1.53 | 1.57 | 1.75 | 1.84 | 6.98 | 6.60 | 5.96 | 5.28 |
| SD | 15.2 | 34.0 | 30.3 | 45.0 | 0.11 | 0.21 | 1.49 | 4.98 | 0.117 | 0.114 | 0.097 | 0.043 | 0.23 | 0.23 | 0.24 | 0.23 | 1.23 | 1.30 | 1.45 | 1.02 |
| 11 | 40.0 | 52.4 | 93.0 | 86.2 | 0.17 | 0.27 | 0.51 | 0.44 | 0.378 | 0.363 | 0.385 | 0.410 | 1.80 | 1.70 | 1.65 | 1.58 | 6.80 | 6.17 | 6.35 | 6.47 |
| 12 | 70.3 | 62.1 | 76.0 | 158.0 | 0.41 | 0.45 | 0.41 | 0.38 | 0.446 | 0.455 | 0.436 | 0.432 | 1.66 | 1.56 | 1.57 | 1.48 | 7.40 | 7.01 | 6.71 | 6.39 |

[1]Early follicular phase.
[2]Late follicular phase.
[3]Early luteal phase.
[4]Late luteal phase.

In Table 1 are also indicated the steroid levels in the 2 patients with hormonal changes not suggestive of ovulation or corpus luteum formation. The plasma T did not show the expected normal pattern presenting circulating levels above the upper limit of control normal cycles at the expected late luteal phase (LL). On the other hand, the %FT presented its lowest values at the LL. Thus, FT remained relatively constant throughout the study in case 11 but it decreased in the 2nd half of the cycle in case 12, in both patients the values however were still within the normal range at the expected LL.
The relation between the progesterone levels and %FT in the plasma samples and pools in which progesterone was added are indicated in Figure 1, where it can be seen that the slopes of the regression lines are variable. However, in all the analysis of variance of the regression coefficients showed them to be statistically significant (9). Probability values (p) are indicated in Figure 1.

## DISCUSSION

A progressive decline in mean total T from early follicular to late luteal phase are indicated by our data, while several investigators have shown that total plasma T levels increase during the first half of the cycle to a peak value at midcycle and decline afterwards to lower levels at the luteal phase, the nadir values attained at the time of the highest P levels (11-13).
However, we could not find significant differences in total T values within either the follicular or luteal periods but in the whole the luteal levels were significantly lower than those observed in the follicular period.
On the other hand, it has been demonstrated that the levels of unbound steroid correlate well with the mass of total circulating steroid present (13, 14), confirmed by our finding of a positive and highly significant correlation between total and FT ($r = 0.8336$, $n = 40$). Therefore, it should be expected that in the luteal phase the levels of FT should be lower than those found in the follicular period.
However we observed that there was a significant increase in % FT in the luteal when compared to the follicular phase though the total levels of T were decreasing. This could be due to a counteracting effect of luteal progesterone which could displace endogenous testosterone from SHBG (sex-hormone binding globulin) and/or CBG (corticosteroid binding globulin) by competition for the binding sites on that protein(s), since some reports suggested that synthetic progestin administration in pharmacologic doses suppresses SHBG binding capacity (14-16). It should be mentioned that these synthetic progestagens, norgestrel isomers, chemically resemble testosterone far more than progesterone.
There is no evidence that physiological levels of P can displace endogenous T from SHBG considering that P is a very poor competitor for sites on SHBG with an affinity constant less than 1% that of testosterone (17).
A possible explanation for our data could be the displacement of endogenous testosterone by P from its albumin binding since 30% of endogenous testosterone and 79% of progesterone are carried by albumin (17). However, the suggestion that P is able to displace T from its binding proteins, albumin in particular, although consistent with results presented in Figure 1 is difficult to understand in face of current knowledge of steroid-protein binding. Albumin has a very high capacity to bind steroids and even in pregnancy most of the steroid binding sites remain unoccupied (17) so that competition between different endogenous steroids is unlikely. A significant decrease in the albumin level would result in an increase in % FT but this is unlikely to have occurred in our study.
Alternatively, the reduced levels of free and total testosterone during the second half of the cycle, could be due to decreased testosterone production by the ovary. However, Serio et al. (18) found testosterone (and androstenedione, the major testosterone prohormone in the adrenals, ovaries and peripheral tissues) concentrations significantly higher in the ovarian veins of subjects studied during the luteal when compared to the follicular phase while it is known that the adrenal contribution to peripheral T (and androstenedione) is relatively constant throughout the menstrual cycle (11). However, because these authors (18) did only measurements of androgen concentration in ovarian blood, without measuring blood flow, nothing can be said about secretion rates. Therefore, although the cause for reduction in testosterone concentration across the menstrual cycle could be due to the decrease in T binding to its binding proteins, consistent with the experimental data, but inconsistent with the physiological data on steroid - protein binding, we cannot discard the possibility that there is decreased T production during the luteal phase of the normal cycle.
Furthermore, in our two anovulatory subjects the lack of increase in P levels would explain the relatively constant total T, throughout their study. Besides, the %FT did not increase as it should be predicted but rather decreased when compared to the normal late luteal phase with the highest progesterone levels.
Accepting the validity of our method of calculation in the equilibrium dialysis technique (7), the finding of an increased %FT in the luteal phase should reduce total testosterone concentration since the mass of the circulating steroid level is dependent on its bound fraction. This is what we found by an independent and sensitive RIA for total testosterone measurement. A simultaneous reduction in T production could not be however discarded.

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